

**DETECTION OF QUINOLONE RESISTANCE IN STAPHYLOCOCCUS
AUREUS ISOLATES IN A TERTIARY CARE CENTRE**

Dissertation submitted to

THE TAMILNADU Dr. M.G.R Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

Branch-IV



DEPARTMENT OF MICROBIOLOGY

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CERTIFICATE

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ACKNOWLEDGEMENT

First I thank Almighty God for giving me immense strength to carry out the project work at all difficulties.

My heartfelt gratitude to the Dean **Dr. S. Ramalingam**, who had permitted me to carry out work in the department and supported at all levels.

I am extremely thankful **Dr. B. Appalaraju**, Professor and Head of Department of Microbiology who had permitted me to do the study in the department. He has been a source of constant support and encouragement throughout my learning process in the department.

I would like to express my sincere thanks to **Dr. S. Parvathi**, Professor, who has been my guide and helping me at all levels through out my work.

I thank the entire faculty, technicians, laboratory attenders and my fellow postgraduates in the department for all the help rendered to me.

I thank my parents for their great support without which things done would not have been possible.

I thank my husband **Er. R. Velusamy** for being my well wisher guide and source of encouragement throughout my post graduation.

Finally, I thank my son and daughter for bearing with me when I compromised time of theirs for completing my work successfully.

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INTRODUCTION

Staphylococcus aureus is a gram-positive coccus frequently found in the nose, respiratory tract, and on the skin. Although *Staphylococcus aureus* is not always pathogenic, it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning. Infections are produced by virulent strains that induce potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. *Staphylococcus aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases of respiratory tract, CNS and bones toxic shock syndrome, bacteremia, and sepsis. It is still one of the five most common causes of hospital-acquired infections and is often the cause of postsurgical wound infections.¹

Staphylococcus aureus is the single most important cause of bloodstream infections in Intensive Care Unit (ICU) and staphylococci are the dominant species in positive blood cultures. *Staphylococcus aureus* is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life threatening infections, and its capacity to adapt to different environmental conditions. The mortality of *Staphylococcus aureus* bacteremia remains approximately 20–40% despite the availability of effective antimicrobials.² *Staphylococcus aureus* is now the leading overall cause of nosocomial infections and as more patients are treated outside the hospital setting, is an increasing concern in community.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. MRSA is any strain of *Staphylococcus aureus* that has developed, through horizontal gene transfer and natural selection, multi- resistance to beta -lactam antibiotics and Quinolones. The emergence of antibiotic-resistant strains of *Staphylococcus aureus* such as Methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide problem in clinical medicine.

Nosocomial infections are produced mostly by MRSA in patients with poor immune systems, open wounds and devices which are invasive. This situation is common in hospitals, prisons, and nursing homes than the general public. The healthcare-associated MRSA is designated as HA-MRSA and community-associated MRSA is defined as CA-MRSA and LA-MRSA (livestock-associated) reflect this distinction. Methicillin - resistant *Staphylococcus aureus* (MRSA) is a persistent problem in community and health care settings.

Quinolone resistance among *Staphylococcus aureus* emerged quickly, more prominently among the methicillin-resistant strains. Fluoroquinolones can not be used as antistaphylococcal agents effectively and its efficacy was dramatically reduced. The rates of quinolone resistance between MSSA and MRSA differ significantly and reason for this is uncertain. One contributing factor is likely antibiotic selective pressure, especially in the hospital setting, resulting in the selection and spread of the more antibiotic-resistant MRSA

strains.³ These resident, resistant strains then become the reservoir for future infections.

The alarming rise in antibiotic resistance among pathogenic bacteria is a persistent issue in antibiotic therapy in health care and community settings.

The fluoroquinolone class of antimicrobial agents has broad acceptance in hospitalized and community patients, and usage appears to be increasing. Although some members of the class (temafloxacin, grepafloxacin, gatifloxacin and trovafloxacin) have been withdrawn or restricted because of adverse events. New members of fluoroquinolones continue to be developed and approved for treating patients with respiratory tract infections, the single most common group of infections. Fluoroquinolones can be used once or twice a day, oral dosing and this easy way increases its use. As we approach the halfway point of the second decade of fluoroquinolone use, resistance has already emerged in some species of bacteria and some clinical settings.⁴

This study has been undertaken to examine the mechanisms of fluoroquinolone resistance and discuss epidemiologic factors that may have contributed to the prevalence of antibiotic resistance in clinical settings.

REVIEW OF LITERATURE

BRIEF HISTORY:

The discovery of *Staphylococcus aureus* was initiated by the microscopic observation of the pus from infected tissue by Alexander Ogston (1844-1929). In 1882, he named the clustered micrococci "*Staphylococci*," from the Greek staphyle, meaning bunch of grapes. With several experiments on guinea pigs and mice, he also provided evidence that this bacterium caused the abscesses seen in infected tissues and septicemia⁵. However, the presence of *Staphylococcus aureus* was officially described and named after it was isolated in 1884 by Anton Rosenbach, who grew the two strains, *Staphylococcus aureus* ("golden staph," for the golden colonies) and *Staphylococcus albus* (white colonies).

The treatment against infections by this bacterium remained very poor, leading to a high rate of mortality worldwide in hospitals until the introduction of penicillin in the early 1940s⁶. This antibiotic became an incredibly strong weapon to cure several staphylococcus infections; however, by the late 1940s penicillin-resistant staphylococcus outbreaks began to occur. When penicillin was no longer efficient to control staphylococcus infections, the antibiotic methicillin was introduced but as this was used and within the short time until 1961, it became less efficient because of repeated cases of resistance. The bacteria evolving resistance to methicillin was first detected in hospital and these were called methicillin-resistant *Staphylococcus aureus* or known as MRSA. Afterwards MRSA strains were known to be not only resistant to methicillin but also a range of penicillin-like antibiotics (beta-lactams) such as

amoxicillin, oxacillin methicillin, cephalosporins and other agents such as erythromycin, aminoglycosides, sulfamethoxazol-trimethoprim ⁷.

The glycopeptide, vancomycin became the last line of defence against *Staphylococcus aureus* infections. In 2002 there was the first report of MRSA strains with reduced susceptibility to vancomycin, in Japan. Following this finding, vancomycin resistance in enterococci (VRE) was believed to have transferred the genes conferring vancomycin resistance to *Staphylococcus aureus*.⁸ The vancomycin resistant *Staphylococcus aureus* strain (VRSA) was first isolated in US (Centers for Disease and Prevention 2002) and then several countries including France, South Africa, Brazil and Korea also reported the presence of VRSA. This issue reflects the global dissemination of these antibiotic resistant bacteria, referred to as “superbugs”. As a result, the requirement for new alternative drugs is urgent; even now for linezolid and teicoplanin that are currently used for the last line of treatment ⁹ there are resistant *Staphylococcus aureus* isolates reported.

MORPHOLOGICAL AND BIOCHEMICAL FEATURES:

Staphylococcus aureus is a Gram-positive cocci facultative anaerobe and is a member of the *Staphylococcaceae* family. More than 30 staphylococcal species are pathogenic, and *Staphylococcus aureus* is the most virulent among them. Its cell wall is from 0.5 to 1.5 µm in size and is naturally arranged in grape-like clusters or groups. It is also a non-motile and non-sporing bacterium. Some strains of *Staphylococcus aureus* are capsulated and

are more virulent than non-capsulated form because the capsular polysaccharide can facilitate the adherence of the bacterium to the host cells and also functions by inhibiting the phagocytosis ¹⁰.

Staphylococcus aureus forms smooth, medium to large colonies which are often yellow. On blood agar medium, the colonies are bigger than those on nutrient agar and most strains are beta-haemolytic ¹¹ and they produce golden yellow pigment on nutrient agar.

Pigmentation:

Carotenoids produced by bacteria are known to act as antioxidants which clear the local reactive oxygen species as shown in several in vitro and in vivo experiments. There are a group of orange and yellow pigments which cause the yellowish colonies for *Staphylococcus aureus*. These pigments are produced from C30 triterpenoid biosynthetic pathway and staphyloxanthin is the end product¹²

In addition, *Staphylococcus aureus* can grow on MacConkey agar, mannitol salt agar. *Staphylococcus aureus* is capable of prolonged survival on environmental surfaces in varying conditions. It can grow on the wide range of temperature (7 to 48°C) and pH (4 to 10) but the optimum temperature is 35 to 37°C and optimum pH is 7 to 7.5. It also can grow in high salt concentrations up to 25%. The bacterial cells are destroyed by heat but the toxin produced by them is extremely heat resistant, it can withstand heat at 60°C in 30 minutes, resulting in the food poisoning and the disease toxic shock syndrome (TSS).

The biochemical properties of *Staphylococcus aureus* are characterized by the fermentation of several sugars such as glucose, sucrose, lactose, maltose and mannitol. Furthermore, it can hydrolyze urea, reduce nitrates to nitrites and liquefy gelatin and it produces DNases. It is positive to catalase, protein A but negative to oxidase, and this is one of the features that can be used to differentiate *Staphylococci* and *Streptococci*.

Catalase:

Catalase is an enzyme used for the decomposition of hydrogen peroxide that is known as a common antimicrobial substance mediated by leukocyte bactericidal mechanisms or by the competitive interference with other species such as Streptococci (*Streptococcus sanguinis*) and Lactobacilli. Catalase can be produced by a number of bacteria but some studies have shown that it is an important virulence factor in these bacteria such as the catalase produced from Lactobacilli can inhibit the growth of *Neisseria gonorrhoeae*. In *Staphylococcus aureus*, catalase has been shown to contribute significantly to the survival in murine macrophages and competition with other pathogens such as *S. pneumoniae* both in vitro and in a murine model of nasal colonisation¹³.

Coagulase positivity is another feature to differentiate between *Staphylococcus aureus* and coagulase negative *Staphylococcus* such as *S. epidermidis* and *S. capitis*.

VIRULENCE FACTORS:

Staphylococcus aureus produces a diverse array of virulence factors including enzymes and proteins involved in surface adhesion and immune evasion. These factors facilitate the pathogenesis of this organism in different stages including colonization of a niche in the host, evasion and suppression of the immune response¹⁴. Based on virulence factors of *Staphylococcus aureus* five groups have been identified.

Group 1:

The enzymes released to lyse cells membranes to facilitate the spread of Infection of the host include

1. Hemolysin (Hla) which lyses the cell membrane of blood cells.
2. Hyaluronidase (HysA) which hydrolyses the hyaluronic acid present in the connective tissue.
3. Staphylokinase (SAK) which breaks down fibrin clots.
4. Lipases (Lip) which hydrolyze lipids on the surface of skin and in subcutaneous tissues in the host ¹⁵.

Group 2:

The surface factors are involved in colonizing host tissues and evading the immune system by inhibiting the phagocytic engulfment or disguising and modulating the immunological system. These proteins are covalently anchored to the cell wall peptidoglycan, thereby are called cell-wall anchored proteins

(CWA). There are 24 identified CWA proteins expressed by *Staphylococcus aureus*, however, their expressions depend on variable growth conditions. Recent reviews have proposed to categorise these CWA proteins into four groups based on their structural motif. These groups of CWA share a basic structure containing a signal sequence at the N-terminus, a wall-spanning region and sorting signal at the C-terminus but differentiate in specific additional regions. The sorting signal has a LPXTG sortase cleavage motif (Leu-Pro-X- Thr-Gly; where X is any amino acid), a hydrophobic domain and a charged tail, involved in covalent anchoring of the protein to the cell wall. Most of CWA proteins are involved in bacterial adhesion to different biomaterial surfaces and immune evasion whereas a subset of these promotes the formation of biofilm.

In addition, these CWA proteins directly or indirectly interact with integrins and facilitate the invasion of non-phagocytic host cells. Intracellular bacteria can cause host cell apoptosis or necrosis, or they can enter a non-disruptive semi-dormant state, including small colony variants (SCVs) ¹⁶. In addition, capsule production is the major surface factor contributing to the inhibition of phagocytosis. More than 90% of *Staphylococcus aureus* clinical strains have been shown to possess several types of capsular polysaccharides in which type 5 and type 8 are relevant in human infections.

Group 3:

The secreted proteins are involved in the suppression of immune cells (Immunoglobulins, complements or neutrophils) or in the inactivation of antimicrobial molecules (lysozymes, defensins). They include:

1. Staphylokinase (SAK) which has been demonstrated to inhibit defensins and opsonophagocytosis.
2. The innate immune modulators including chemotaxis inhibitory protein (CHIPS).
3. The staphylococcal complement inhibitor (SCIN) which is employed to inactivate the human complements ¹⁷.
4. O-acetyltransferase (OatA) released from the cell wall combining.
5. Wall teichoic acids (WTA) which have been shown to play a role in deactivating host lysozyme activity.

Group 4:

Toxins involving in septic shock include:

1. Exotoxins (e.g. hemolysins with four different types - alpha, beta, gamma and delta - which all lyse the membrane of blood cells).
2. Leukotoxin (LukSF), leukocidins (LukED-PVL) which kill polymorphonuclear leucocytes and macrophages and cause dermonecrosis.
3. Enterotoxins (with eight antigenic types from A to H; responsible for food poisoning); enterotoxins SEA to SEG (EntA, EntB, SeA-G) act as

superantigens stimulating the proliferation of T-cells, these toxins are the leading cause for Staphylococcal gastroenteritis.

4. Toxic shock syndrome toxin (TSST-1) which causes a life-threatening pathology characterized by fever, hypertension, vomiting, diarrhoea and erythematous rash.

5. Exfoliative toxins (responsible for Staphylococcal scalded skin syndrome¹⁸.

Group 5:

Bacteriocins are defined as antimicrobial molecules with a broad-spectrum activity and involved in microbial competition with different species in colonization and infections. *Staphylococcus aureus* is able to produce several types of bacteriocins, in particular, a group of lantibiotics is well characterised. The lantibiotics are a group of ribosomal synthesised, post-translationally modified peptides containing unusual amino acids, such as dehydrated and lanthionine residues. This group of bacteriocins has attracted much attention in recent years due to the success of the well characterised lantibiotic, nisin, as a food preservative.¹⁹

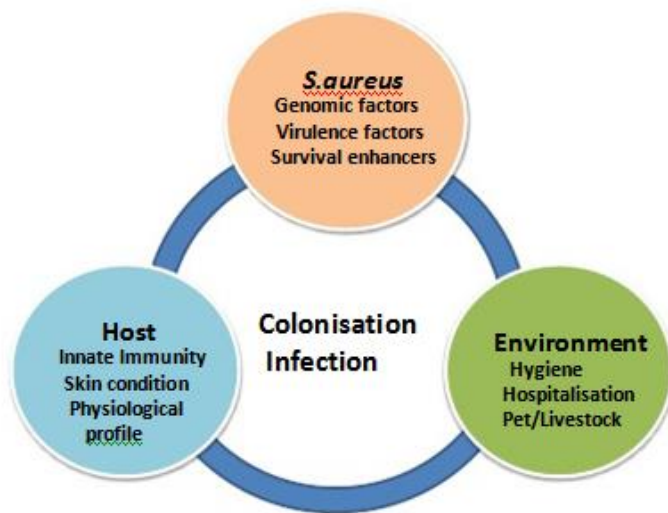
Lantibiotics are defined as small peptides less than 5 kDa comprising of two main unusual amino acids lanthionine (Lan), L- methyllanthionine (MeLan) and a number of dehydrated amino acids such as the α , β - unsaturated amino acids Dha and Dhb ²⁰. There are two groups of lantibiotics including Type A and Type B that can be differentiated by their structure and function.

Type A lantibiotics including epidermin, nisin, subtilin are characterised by the long peptide chains with 34 residues and damage the cell membrane of competitive organisms. Type B lantibiotics have a globular structure with 19 residues and play a role in deactivating enzyme in cell wall biosynthesis. These exoproducts target other strains of *Staphylococcus aureus*, coagulase negative *Staphylococci* (*S. epidermidis*), *Corynebacteria* (*C. pseudodiphtheriticum*) and *Streptococci* (*S. pneumoniae*).

PATHOGENESIS:

Staphylococcus aureus is an opportunistic pathogen which only switches to be pathogenic under some circumstances. Some people are intermittent / persistent carriers of *Staphylococcus aureus* as a normal flora but others are not. Some individuals can carry *Staphylococcus aureus* for many years but may not develop disease. The higher risk of infection among persistent carriers than in intermittent and non-carriers has been previously described. *Staphylococcus aureus* infections occur when this organism invades the host and evades the host defence successfully, and this requires some initial steps such as inoculation and local colonisation of tissue surfaces. To survive in the host, *Staphylococcus aureus* must adapt to different stress factors such as nutrient limitation, desiccation, and changes in temperature, osmolarity and pH, competition from other bacteria as well as the antimicrobial actions of the human body²¹.

The interactions among the host, bacteria and environmental factors contributing to the invasion of *Staphylococcus aureus*. Of these factors, the host factors are thought to play a key role and bacterial factors are thought to determine which strain is carried²².



The interplay between bacterial, host and environment factors involved in *Staphylococcus aureus* colonization and infection. (Peacock et al. 2001; Lowy 2011)

BACTERIAL FACTORS:

Bacterial factors play an important role in colonisation and infections because some *Staphylococcus aureus* strains are observed in disease states more frequently than others. Bacterial factors are hallmarked by the diversity between *Staphylococcus aureus* genomes and the virulence factors, and factors specified for colonization and immune suppression.

GENOMIC FACTORS:

The *Staphylococcus aureus* genome varies from 2.5 to 3.1 Mb in size, and contains around 2,500 open reading frames. Numerous strains of *Staphylococcus aureus* have been sequenced such as N315 and Mu50, MW2 MRSA252 and MSSA476, COL Newman, and the comparison data between these sequenced strains has shown a significant difference in some features among *Staphylococcus aureus* strains²³.

The in silico analysis on *Staphylococcus aureus* genomes revealed it consists of three main parts:

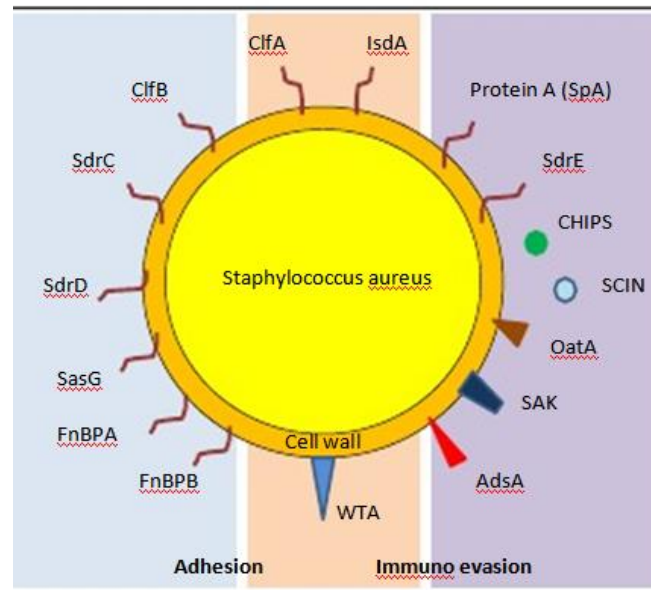
1. Core genes (~ 80%, genes that have been highly conserved among isolates) encoding surface proteins promoting the *Staphylococcus aureus* adhesion and metabolic/regulatory proteins.
2. Core variable genes (~10-12%, genes that vary or missing between genomes), encoding regulators of virulence genes or surface proteins involving in host interactions;
3. Mobile genetic elements (MGEs) (~10-20%, genes that can horizontally transfer among isolates) consisting more than 50% of virulence factors in *Staphylococcus aureus* including bacteriophages, pathogenicity islands, transposons, insertions, genomic islands, plasmid, etc.²⁴. The MGEs allow the exchange of virulence factors and antibiotic resistance features among isolates, possibly resulting in new combinations of virulence factors and antibiotic resistance determinants. This issue may be responsible for the emergence of

new strains harbouring more virulent or more resistant combinations in the community.

The differences in genomic contents among the sequenced strains had been determined in the core genes but these are not as significant as in the MGEs region. The distinctive MGEs may support the versatile adaptation of strains specialised for the infection of selected host tissues²⁵.

HOST FACTORS:

The role of host factors in the outcome of *Staphylococcus aureus* infections have been extensively studied in different infections such as in rhinosininitis, osteomyelitis, arthritis and endocarditis.²⁶ These factors comprise numerous immunological cells which are involved in different stages of colonisation and infections such as neutrophils, macrophages, B cells, T cells, Natural Killer Cells, MHC Class II, interleukin-4, interleukin-12, complement factors, IFN- γ (interferon), TNF (tumour necrosis factor). These immune cells will act as the barriers to prevent the invasion of *Staphylococcus aureus*. In order to survive, this pathogen possesses numerous CWA proteins which are important for both adhesion and immune evasion.



Nasal colonisation and the functions of CWA proteins present in *Staphylococcus aureus*. (Nestle et al. 2009; Foster et al. 2014).

During nasal colonisation, *Staphylococcus aureus* adheres to the epidermal cells which comprise 4 layers including stratum corneum, stratum granulosum, and stratum spinosum and stratum basale. The immune cells such as Langerhans cells in the epidermis and other cells such as natural killer (NK) cells, macrophages, T-cells, B- cells, mast cells, dermal dendritic cells and plasma cells in the dermis will act as the barriers to prevent the invasion of *Staphylococcus aureus*. This pathogen possesses numerous CWA proteins including several adhesion factors (blue background) as well as factors involved in immune evasion (purple background), and some factors which are important for both adhesion and immune evasion (orange background). These CWA proteins include CHIPS: chemotaxis inhibitory protein of *Staphylococcus aureus*, SCIN: Staphylococcal Complement Inhibitor; OatA:

O-acetyltransferase; SAK: staphylokinase; WTA: wall teichoic acid; SasG: surface protein G; SdrE, SdrD, SdrC: serine-aspartic acid repeat protein E, D and C; FnBPA, FnBPB: fibronectin-binding proteins A, B; ClfA, ClfB: clumping factor A, B; IsdA: iron-regulated surface determinant, AdsA: Adenosine synthase.^{27, 28}

In recent investigations, there are specific host genetic factors that have been suggested to be important determinants for persistent nasal carriage and infections of *Staphylococcus aureus* in humans such as the presence of single nucleotide polymorphisms (SNPs) in the glucocorticoid receptor gene²⁹, the serine protease C1 inhibitor as well as in interleukin-4. Furthermore, the skin damage, open wound, cuts and lesions as well as the physiological conditions of various niches (limited nutrients, limited oxygen, fluctuated osmolarity, pH, the presence of oxidative, nitrosative stress or other toxic agents) may facilitate the colonisation and infection of specific *Staphylococcus aureus* strains or contribute to the phenotype switching.

ENVIRONMENT FACTORS

Environmental factors increase the risk for carrying *Staphylococcus aureus*, as determined in numerous clinical reports, such as the crowding and the hygiene conditions in both health care facilities/professionals, and in household setting/family members. Moreover, pets/livestock might become reservoirs of *Staphylococcus aureus* and serve as intermediates in the transmission cycle³⁰.

The interplay between bacterial factors, host factors and environmental factors seems to be crucial for the *Staphylococcus aureus* colonisation and infection process. *Staphylococcus aureus* has been previously known as an extra cellular organism, however, it also can be found to survive and persist in various host tissues including professional phagocytes and non professional phagocytes such as epithelial cells, osteoblasts³¹, endothelial cells, fibroblasts, and keratinocytes. This incredible ability of survival by this bacterium shows that *Staphylococcus aureus* is a versatile pathogen that can employ several strategies to be undetected by the host immune system or for inactivating the immune response by using a plethora of virulence factors related to colonisation, invasion and immune evasion.

STAPHYLOCOCCUS AUREUS INFECTIONS AND DISEASES:

Staphylococcus aureus causes a wide range of illnesses through the body due to its capacity to colonize and grow in different kinds of host tissues. Anterior nares and skin are the frequent sites that *Staphylococcus aureus* colonizes as a harmless commensal organism but it can be also found in several body sites such as axillae, vagina and the gastrointestinal tract³². Approximately 20-25% of the human population are long term carriers, and 75-80% are intermittent carriers or non carriers of *Staphylococcus aureus*.

However, the rate is higher among immune- compromised patients such as insulin-dependent diabetes, HIV- infected patients and patients undergoing

hemodialysis or skin damage conditions. The minor cutaneous infections caused by *Staphylococcus aureus* include carbuncles, boils, impetigo, burns, surgical site infections and wound infections. The more severe infections and life threatening infections such as sinusitis, tonsillitis, osteomyelitis, pneumonitis, endocarditis, meningitis, and bacteraemia occur when *Staphylococcus aureus* enters the body via an opening cut or wound. Additionally, by releasing the toxins on the food or in the blood stream, *Staphylococcus aureus* can also cause food poisoning, scalded skin syndrome and toxic shock syndrome³³.

Clinical data have indicated that *Staphylococcus aureus* is the primary cause of lower respiratory tract infections and surgical site infections and the second leading cause of nosocomial bacteraemia, pneumonia, and cardiovascular infections. It is also the leading cause of primary septic arthritis and osteomyelitis in all ages except neonates³⁴. However, in one global study spanning 100 hospitals in Canada, US, Latin America, Europe and West Pacific, *Staphylococcus aureus* has been demonstrated as the most prevalent cause of nosocomial bloodstream, skin and soft tissue infections (SSTIs) and pneumonia³⁵. *Staphylococcus aureus* infections are usually developed in human by autoinfection – individuals being infected with their own colonising strains. However, individuals can acquire *Staphylococcus aureus* from other infected individuals via direct skin to skin transmission and nasal secretions or from environmental exposures such as humid conditions, public places, and lack of hygiene. The spreading of *Staphylococcus aureus* infections in the

community involving the CA-MRSA clones (community - acquired MRSA) whereas HA-MRSA clones (hospital-associated MRSA) dominate the health care facilities and pose both a huge financial cost and threat of serious infection for patients and the community³⁶.

Staphylococcus aureus contamination in the environment in hospitals (such as ventilators and bed ledges in intensive care unit) is the major reservoir for crossing transmission between patients, medical devices and health care staff. The control of HA-MRSA infections is well managed in health professionals who are potential carriers of HA-MRSA. However, patients, in particular if immune-compromised, have a higher risk of infection (30-60%) and it remains near- impossible to clear or control the invading bacteria³⁷. *Staphylococcus aureus* is also known to colonise and infect both pets and livestock. *Staphylococcus aureus* infection in humans is always a major concern but *Staphylococcus aureus* - infected animals (especially MRSA) could be the important reservoirs for human colonisation, leading to a potential pandemic.

EPIDEMIOLOGY:

The emergence of infections associated with *Staphylococcus aureus* has been alarming mainly due to its resistance to multiple antibiotics. In US, the hospitalisation and mortality rate of the infections caused by *Staphylococcus aureus* is approximately twice the length of stay, deaths and medical costs of typical hospitalisations. Importantly, the MRSA rates have been increasing

rapidly worldwide during the last decades. Patients with MRSA infections have a 2.5-times higher average attributable death rate compared to MSSA infection³⁸. Infections with *Staphylococcus aureus* are especially difficult to treat because of evolved resistance to antimicrobial drugs. Numerous clinical experts believe that the dissemination of MRSA through the community is as a result of unreasonable use of antibiotics in treatment, this selective pressure thereby leading to the rapid evolution of drug resistance in *Staphylococcus aureus* strains.

The multi- drug resistant *Staphylococcus* strains currently known have become widespread in both healthcare facilities and the community and in both developed and developing countries. According to the SENTRY Antimicrobial Surveillance Program, between the years of 1997 and 1999, MRSA prevalence was 23% (of all *Staphylococcus aureus* strains) in Australia, 26% in Europe, 32% in the USA, 35% in Latin America, 40% in South America, and 67% in Japan³⁹. In one study in US, the death rates by Community - acquired and nosocomial *Staphylococcus aureus* infections were similar but *Staphylococcus aureus* - associated hospitalizations resulted in approximately twice the length of stay, and medical costs per patient⁴⁰.

HA-MRSA infection is globally alarming because various HA-MRSA clones have spread worldwide and caused the high rate of morbidity and mortality. Indeed, a 2007 study in US estimated the number of deaths due to HA-MRSA infections in 2005 was 17,000 which surpassed those from AIDS. The cost of treating patients with MRSA is estimated between a \$4 billion to

\$30 billion per year burden for the US economy⁴¹. One Canadian study stated the MRSA finance burden in Canada ranged from \$40 to \$59 million in 2000. Although HA-MRSA is an obvious concern recently, the CA-MRSA infections have been reported with increasing prevalence. A study performed in emergency departments in 11 cities in US found that 78% of the isolates were MRSA, and among these, 98% were the CA- MRSA USA300 strain, one of the most common strains of MRSA⁴².

MOLECULAR BASIS FOR ANTIBIOTIC RESISTANCE:

From genomics studies, the acquisition of *Staphylococcus aureus* resistance in MRSA has been assigned to the presence of the gene ‘mecA’ located on the *Staphylococcal* chromosome cassette mec (SCCmec); a novel, mobile resistance element. The gene mecA encodes the 78-kDa penicillin binding protein 2A (PBP2A) which has a low affinity for β -lactam antibiotics leading to the inhibition of cell wall synthesis by inactivating transpeptidase. The regulation of mecA is controlled by the repressor MecI and the transmembrane β -lactam-sensing signal-transducer MecR1, which are both divergently transcribed. The integration of SCCmec into the genome is carried out with the cassette chromosome recombinases (ccr) genes including ccrA, ccrB and ccrC that are located on all SCCmec elements at a specific site. These genes excise and integrate at the SCCmec attachment site (attB_{sc}) at the 3’ end of an open reading frame (orfX)⁴³.

There are eleven subtypes of SCCmec from I to XI and six classes (A, B, C1, C2, D and E) performing different resistance patterns based on the arrangement of mec complex genes including mecA, regulatory genes mecI and mecR1 and with the insertion sequences⁴⁴. Class A is identified with the presence of a complete mecR1 and mecI upstream and the variable regions and insertion sequence IS431 downstream of mecA. Class B is characterized by the presence of a truncated mecR1 (Δ mecR1) and insert sequence IS272 upstream whereas the variable regions and insertion sequence IS431 downstream. Class C is slightly different from Class B in that the presence of IS272 upstream is replaced by IS431. Based on the orientation of upstream and downstream IS431, there are two sub- classes C1 (same orientation) and C2 (reversed). Class D is different to Class C in that there is no presence of insertion sequence in the downstream region of Δ mecR1. One new arrangement has been classified as SCCmec type XI and class E in which mecR1LGA251 and mecILGA251 are located upstream of mecALGA251 and the blaZ gene is located downstream.

Recent articles have shown that CA-MRSA evolves independently from HA- MRSA. CA-MRSA strains usually have different subtypes of SCCmec (IV,V,VII), are often resistant to fewer antibiotic classes (frequently only β -lactams and macrolides), and are more virulent, with a high proportion carrying the genes encoding Panton -Valentine leukocidin (PVL). The presence of PVL is suggested as a feature to differentiate HA-MRSA and CA-MRSA, but this is still in question. Differently, HA-MRSA possesses the larger SCCmec subtypes

(I, II, III) containing multiple-drug resistance determinants, leading to slower growth rate than CA- MRSA due to the heavy metabolic burden associated with the large size of SCCmec. However, with the dissemination of CA-MRSA in hospitals, the distinction between CA-MRSA and HA-MRSA seems to fade. Currently eleven SCC mec types in *Staphylococcus aureus* strains based on their gene arrangements.⁴⁵

| SCC mec type | Ccr gene complex | Mec gene complex |
|--------------|-----------------------|------------------|
| I | 1 (A1B1) | B |
| II | 2 (A2B2) | A |
| III | 3 (A3B3) | A |
| IV | 2 (A2B2) | B |
| V | 5 (C) | C2 |
| VI | 4 (A4B4) | B |
| VII | 5 (C) | C1 |
| VIII | 4 (A4B4) ^b | A |

Classification of Staphylococcal Cassette Chromosome mec (SCCmec)

Antimicrobial Agents and Chemotherapy, Dec. 2009, p. 4961–4967.

NOVEL BACTERIAL LIFESTYLES AS STRESS RESPONSES:

To adapt to different environmental conditions outside and inside the host, some bacteria are capable of switching from their normal lifestyles to form quiescent phenotypes such as biofilms and SCVs. These quasi-dormant lifestyles have been characterized by a slower growth rate but with a prolonged survival capacity in the host both extracellularly and intracellularly when compared to planktonic cells. The existence of these alternative lifestyles in clinical settings of *Staphylococcus aureus* has been detected when the rate of chronic or relapse infections has drastically increased although the various antibiotic regimes have been applied⁴⁶.

Compared to the planktonic lifestyle of *Staphylococcus aureus*, biofilms and SCVs cells have been firstly revealed only since 1976 (biofilm) and since 1955 (SCVs) in clinical settings. After some decades, an understanding related to the formation, the regulation, the pathogenesis as well as the detection and control of biofilm and SCVs is beginning to made, but there are still unclear and conflicting issues which require more investigation⁴⁷.

SMALL COLONY VARIANTS:

The development of novel lifestyles such as biofilm and small colony variants (SCVs) has been lately discovered as the main reasons for chronicity and relapse in *Staphylococcus aureus* infections. Several studies indicated and confirmed the crucial role of these switching lifestyles in clinical settings; in

particular, osteomyelitis, arthritis, rhino sinusitis, cystic fibrosis, soft tissue infections, sepsis, endocarditis and medical devices associated infections⁴⁸.

Infections involving either biofilm or SCVs are rarely resolved by host defences because in these states the pathogen is capable of surviving and developing a dormant state within various tissues. When suitable conditions arise the infections will recur unpredictably and often become more serious and even life threatening due to belated detection and treatment. These latent forms of *Staphylococcus aureus* have been causing a huge challenge for diagnosis and treatment, often resulting in higher rate of medical cost and mortality.

Recent investigations have shown the role of *Staphylococcus aureus* biofilms and SCVs is central to relapsing infections. The estimated frequency of occurrence of human *Staphylococcus aureus* SCVs varies between 1 and 30% of clinical samples. *Staphylococcus aureus* SCV was found in 29% of patients with osteomyelitis, 17–46% of patients with cystic fibrosis who were chronically colonised with *Staphylococcus aureus*⁴⁹. There are several clear indications that cystic fibrosis, periodontitis, bloodstream and urinary tract infections result from *Staphylococcus aureus* biofilms indwelling medical device. The evolution in the lifestyle switching of *Staphylococcus aureus* has been extensively investigated and some understanding has been elucidated. However, various questions are still open regarding the formation, the regulation and related metabolic pathways as well as the pathogenesis of SCVs⁵⁰. The key difficulty in the study of SCVs is that when cultured in the laboratory they revert to their parental growth-type.

BIOFILMS:

Biofilms formed by bacteria have been described and researched for decades due to their increasing importance in pathogenesis. Originally, biofilms are known to cause industrial problems and now they are implicated in the spread of device-related and chronic infections. Bacterial biofilms can be isolated from mucosal or tissue samples of cystic fibrosis, native heart valve in endocarditis, as well as in otitis media, rhinosinusitis, tonsillitis patients and prosthetic devices such as central venous catheter tips, urinary catheter, and many other clinical situations. It is easily observed that these patients have optimal rough surfaces for biofilm attachment and growth⁵¹. Regarding pathological aspects, the planktonic form is often responsible for acute symptoms and systemic responses whereas biofilms cause chronic infections but acute exacerbations due to the complex latent immune sequela that they can provoke.

Several pathogenic bacteria such as *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Hemophilus influenzae* and *Staphylococcus aureus* are capable of biofilm formation that are clinically relevant. Numerous studies have indicated that there is a high prevalence of biofilm - mediated infections caused by *Staphylococcus aureus*. Importantly, its persistence and antibiotic resistance, have widely been reported in clinical outcomes worldwide. A recent study over a large number of clinical isolates has revealed that the strains with greater multi-resistance to antimicrobial compounds have significantly

increased their ability for biofilm formation rather than those with less resistance ⁵².

DEFINITION AND FORMATION OF BIOFILMS:

According to the current definition, biofilms are characterized by heterogeneous Multi - layers of sessile single cells and micro-colonies, which are encased in a matrix of extra cellular polymeric substances (EPS). This matrix is possibly composed of intercellular polysaccharide adhesions (PIA) and proteins along with extra cellular genetic materials (such as extra cellular DNA). These layers cover and attach to a substratum, interface or to each other persistently and thereby establishing a recalcitrant block to resist external destructive factors. Within biofilms, there are four distinct metabolic states of growing cells including aerobically (often located in the outmost layers exposed to oxygen and nutrients) fermentative, dormant and dead in which the dormant cells are dominant and lodge in the anoxic layers ⁵³.

The changes in cell-type due to the phenotypic change, the altered growth rate as well as the expression of genes related to the cell's metabolic pathways, surface structures and virulence factors have all been well-documented. The formation of biofilms has been proposed to consist of four phases namely attachment, accumulation, maturation, and dispersal.

Attachment and Accumulation

In the first stage, the attachment of planktonic cells depends on the surface appendages. The presence of high shear forces, for instance, in mucous-covered surfaces, will promote the initiation of biofilm formation. In the stage of aggregation, the surface factors including a variety of CWA proteins and extracellular DNA (eDNA) and teichoic acids (TA) have been shown as crucial components for the bacterial adherence for *Staphylococcus aureus*. When the adjacent cells interact, the structure of some CWA proteins (fibronectin-binding proteins (FnBPs) and SasG) may be modified and twist around each other or these can bind to other ligands on neighboring cells to promote the aggregation of cells⁵⁴.

Maturation

When a number of cells are grouped and bonded based on the change in their cell wall structures. The main molecule responsible for the aggregation in *Staphylococcus aureus* is polysaccharide intercellular adhesin (PIA), which composed of N- acetylglucosamine residues; 20% of the residues are deacetylated and are thus positively charged. However, PIA-mediated biofilms is not unique, some strains isolated from biofilm- associated infections do not have the *ica* genes. In these PIA-independent biofilms, the cell wall-anchored proteins (CWA) are more relevant. In a maturation stage, biofilm structure has been described as “towers” or “mushrooms” containing a system of fluid-filled channels which is believed to be responsible delivering nutrients to cells to

deeper layers. Nevertheless, the most recent publication has revealed that the eDNA released from lysis cells is a predominant component in biofilm maturation of *Staphylococcus aureus*⁵⁴. As a polyanionic molecule, eDNA plays an important role in shaping the matrix is due to the capacity to link other ingredients together and facilitate the biofilm adhesion to surface well.

Dispersal

As the biofilm is completely developed, single cells or small clusters of cells are able to detach from the mature biofilm and reallocate to other distant sites. The mechanisms involved in the dissemination of biofilms are still unclear. The biofilm dispersal is probably relevant for the spread of biofilm-associated infections and these issues can be observed in clinical cases such as endocarditis (bacterial detachment from biofilms on heart valves), pneumonia (bacterial dispersal from biofilms from endotracheal tube / oropharynx) or sepsis⁵⁵.

Some significant features for dissemination have been recently uncovered, including extracellular bacterial products which degrade and solubilise the adhesive component of the matrix, environmental conditions, and polymicrobial interactions. Indeed, the production of extracellular enzymes (DNases, proteases) and surfactants (phenol soluble modulins - PSMs) have been indicated as the primary factors which promote the detachment of bacterial cells from biofilm matrix. The deletion of the genes encoding the

proteases and the addition of protease inhibitors both resulted in a significant increase in *Staphylococcus aureus* biofilm formation. Similarly, the nuclease-deficient mutant strains of *Staphylococcus aureus* and the addition of DNases exhibited significantly increased biofilm formation, indicating that eDNA is a major part of the biofilm matrix. In addition, PSMs have been demonstrated to promote biofilm disassembly in vitro and promote the bacterial dissemination from colonised catheters in a mouse model of device-related infection. PSMs are surfactant-like peptides due to their amphiphilic helical structures that are regulated by the agr quorum-sensing system⁵⁵. So far little detail is known about the impact of environmental conditions and polymicrobial interactions on biofilm dispersal.

The accessory gene regulator (agr) quorum-sensing system is involved in dissemination of bacterial cells from biofilms. When the biofilm is mature, the presence of auto-inducing peptides (AIPs) throughout the bacterial community can reach a quorum sensing threshold and leads to the induction of the expression of agr. The up-regulation of agr induces the formation of PSMs, protease and nuclease expression, leading to the detachment of planktonic cells out of biofilm matrix to relocate in other host tissues. The agr mutants formed a thicker and more compact biofilm in vitro compared to isogenic wild-type strains⁵⁶. Most likely, the permanent disabling of agr regulation and the consequent excessive biofilm formation are of advantage to bacterial survival in specific stages or types of infection. Notably, mutations that produce agr-

negative phenotypes are common and can also be seen in vitro where they occur at a high rate.

In general, during infections, the early step of accumulation and attachment is a prerequisite for the bacterial colonisation on host niches or tissues or on implanted and prosthetic medical devices, whereas the final detachment is a crucial role for the dissemination of an infection. In addition, the composition of biofilm matrix is possibly variable among the *Staphylococcus aureus* strains; it seems to rely on various environmental conditions of host tissues or niches with changeable physiological and physical conditions as well as the availability of nutrients. The role of PIA in the *Staphylococcus aureus* biofilm matrix is still an area of some conflict as some studies indicated that it is not crucial for the development of biofilm compared to the results from previous studies. The understanding of the complexities of the Staphylococcal biofilm matrix remains incomplete and requires more research in the future.

DEFINING CHARACTERISTICS

Staphylococcus aureus SCVs have a distinct phenotype which is characterized by a slow growth rate and atypical morphological and biochemical properties compared to the parental phenotype. Therefore, SCVs have just been defined after incubation after 48 or 72 hrs to form visible colonies on the solid agar plates. Their size of colonies are very small as pin-

point, or can be defined as less than 1/10th in size compared to normal size of *Staphylococcus aureus* colonies. In addition, the pigment of colonies is significantly reduced or colourless and their hemolysis is greatly reduced or negative.

Recent research have added a variety of new characteristics for SCVs, however, these findings may only be indicative for SCVs recovered from the specific strains or isolates within those studies. According to some authors, SCVs are auxotrophic for hemin, menadione and/or thymidine and an altered ability to utilise different carbon sources such as glucose and fructose but not able to use mannitol or other sugars such as turanose and lactose. Other reports showed that SCVs have reduced coagulase production and require more than 18 hrs of incubation to be coagulase positive. SCVs have been often represented in a mixed population with normal *S. aureus*, furthermore, SCVs may revert to the (wild type) WT when growing together in the rich medium without adding antibiotics, this causes *Staphylococcus aureus* SCVs to be frequently unidentified. In addition, the slow growth rate may affect the diffusion test or other antibiotic susceptibility tests, leading to the misidentification when using standard clinical microbiology procedures ⁵⁷.

METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

In the year 1942, the first penicillin resistant *Staphylococcus aureus* was identified. All the resistant strains become pandemic in early 1950s. Resistance to Methicillin and vancomycin developed in most strains of *Staphylococcus aureus* is mediated by *mecA* and *van A* genes respectively. They are also resistant to β -lactam antibiotics and classes of antibiotics. European hospitals reported MRSA infections that caused an outbreak during early 1960s. About 25% of nosocomial infections in health care institutions are caused by MRSA infections in US with half of associated deaths. High mortality has also been associated with MSSA that caused death mainly due to septicemia. In spite of use of appropriate chemotherapy most of serious sequelae are due to MRSA than MSSA and overall mortality rate continues to rise.

The transmission of bacteria takes place by carrier colonized with bacteria, IV drug addicts, Insulin dependant diabetes and those with intravascular catheters for prolonged period constitute high risk group. Higher colonization rate has been observed in children due to their frequent contact with respiratory secretions. Emerging MRSA strains confined not only to hospital but they also appear in community due to inappropriate use of antibiotics which caused overgrowth and increased pathogenicity of the organism.

Due to evolutionary changes and epidemiological expression of the MRSA strains, new MRSA strains have now emerged which differ from the initial isolates. Beyond antibiotics the ability of the organism to adapt to the

environment and advances in patient care causes MRSA emerging as a major pathogen not only in hospital but also in community. Person to person transmission has been reported in CA-MRSA infection hence it is associated with higher morbidity and mortality. The knowledge of circulating MRSA clones under the prevailing scenario can be helpful in controlling transmission and optimizing treatment⁵⁸.

After 1990s people who were not hospitalised but undergone invasive procedure MRSA has become a matter of great concern. Infections caused by such strains are called as CA MRSA. High risk populations such as intravenous drug users, patients in nursing homes, chronically ill person, CA MRSA first reported but also found in healthy children. Till recently these strains are sensitive to all antibiotics other than b lactams but now resistance to most of the antibiotics has emerged. The clonal lineages of *Staphylococcus aureus* have tendency to colonize specific species and may adapt either human or animals. The more prevalent epidemic strains are capable of spread with in hospital or to other places outside but the sporadic stains spread widely. Extended host spectrum genotypes have appeared which have different lineages, host specificity and tendency to infect wide range of species.

For example MRSA ST22-IV, EMRSA15 have been reported in pigs (rarely), turtles, bats, cats, dogs, and humans⁵⁹. In US hospitals, most commonly identified antibiotic resistant pathogen is MRSA and 25.9% of *Staphylococcus aureus* strains isolated from out patients were methicillin resistant (National infectious surveillance system report, 2003). Most of these

strains were isolated from persons who acquired them from healthcare environment.⁶⁰

The MRSA is defined by presence of *mecA* and antibiotic susceptibility is used by some researchers to identify it. A genetic evolutionary analysis demonstrated that the *mecA* gene has been transferred into methicillin-susceptible *Staphylococcus aureus* (MSSA) more than 20 times and emerged in more than 5 phylogenetically different lineages as well as re-emerged within individual lineages. The introduction of the *mecA* gene from putative donor species into MSSA strains which are already adapted successfully to the hospital environment and to the community, created successful epidemic HA-MRSA and CA-MRSA clones. It is believed that ancestral MRSA genotypes, ST250- MRSA originated in Denmark which possesses SCCmec type I, were recovered in the 1960s.

This genotype arose as it acquired the *mec* gene by ST250-MSSA, which itself had arisen from ST8-MSSA by chromosomal point mutation⁶¹. ST250-MRSA evolved by a single point mutation, an important hospital pathogen in Europe and has been reported to cause outbreaks in New York hospital. The emergence of CA-MRSA strains has repeatedly occurred as a result of the introduction of SCCmec type IV into a different genetic MSSA background. MDR usually seen in HA-MRSA strain and antibiotic resistance is limited to beta-lactams in CA-MRSA. The size of SCCmec type IV is small which prevents its carriage of additional genetic material, in contrast to the

characteristic presence of additional genetic material in SCCmec type II and SCCmec type III.

INTRODUCTION TO FLUOROQUINOLONES:

In 1960s Nalidixic acid was used a drug of choice for most of Gram negative bacteria but it is less effective on Gram positive bacteria and frequent daily administration and tendency to cause photosensitivity reactions and convulsions limited its use.

In mid 1980s introduction of norfloxacin followed by ciprofloxacin was a major improvement over Nalidixic acid. The advantage of twice daily administration and ability to obtain high concentrations in tissues and fluids with high clinical efficacy with excellent activity against Gram negative and some action on Gram positive bacteria made it as a good drug.

The new fluoroquinolones have a substituent of carboxylic acid at position 3 and at position 4 have a carboxyl group which helps in transport into bacterial cells and attachment to the DNA gyrase complex which are significantly done by these groups.

In vitro potency, mostly against Gram-positive bacteria is almost determined by the amino substituent at position 5 of the quinolone ring, as found in sparfloxacin. Grepafloxacin enhances potency against Gram positive bacteria because of presence of methyl group at C-5 to a lesser extent. A fluoro substituent, present at C-6 position in all new fluoroquinolones is responsible for fluoro nomenclature and increases antibacterial potency⁶².

The presence of piperazine ring at position 7 increases efficacy of fluoroquinolones against Gram negative organisms (eg: ciprofloxacin, gatifloxacin, grepafloxacin, levofloxacin and sparfloxacin) and pyrrolidine ring at the same position enhance activity on Gram positive bacteria alkyl group of both ring type increases solubility prolongs the half life and excellent action on Gram positive bacteria, Ciprofloxacin and grepafloxacin interact with theophylline because of presence of non bulky side chains at C-7.

Presence of halogen at position 8 widens the spectrum of antibacterial activity and extends action on anaerobes in sparfloxacin and clinafloxacin similarly methoxy substituent at position 8 in gatifloxacin and moxifloxacin enhance activity on anaerobes.

MECHANISM OF RESISTANCE TO FLUOROQUINOLONES:

The enzymes topoisomerase and gyrase control DNA levels and play important role in nucleic acid processes. They pass an intact double helix via separate segment of DNA by generating a transient double stranded break. The two distinct functional subunits of Gyrase and topoisomerase are gyrA and gyrB and homologous subunits grlA and grlB respectively in Gram positive species⁶³.

The active site that is present in gyrA is tyrosine residue. gyrB has the ATPase domain and also TOPRIM domain, both of them binds the divalent metal ions that are responsible for DNA cleavage and ligation. Gyrase and topoisomerase IV have different functions of introducing negative super coils

inside the DNA physiologically in spite of their structural similarities gyrase belongs only to type II topoisomerase. The ω protein (a type I topoisomerase) combines and works with gyrase to form the super helical density of the bacteria chromosome. The torsional stress is removed primarily by the gyrase enzyme.

Chromosomal superhelical density and alleviating torsional stress is less commonly maintained by topoisomerase IV than gyrase and taking away the knots from the bacterial chromosome due to basic cellular process removing daughter chromosomes as a result of replication is the major function of this enzyme.

In *Staphylococcus aureus* the flouroquinolone resistance mechanism is mainly due to substitutions of aminoacid at particular locations in the DNA gyrase A and B subunits as well as in the topoisomerase IV subunits and also from norA mediated efflux .In a single strain more than one resistance mechanism can be present⁶⁴.

Disk diffusion method by Kirby Bauer Method:

The sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to multiple antimicrobial compounds is determined by the Kirby-Bauer disk diffusion susceptibility test. Mueller-Hinton agar is used to grow the pathogenic organism in the presence of various antimicrobial impregnated filter paper disks. The ability of the antibiotic to inhibit growth is determined by

presence or absence of growth around the disk. A filter paper disk of about 6mm is placed on Muller Hinton agar plate. The water present on the disk will be absorbed immediately⁶⁵. The antibiotic starts to diffuse in to the medium and the concentration will be more near the disk it reduces gradually away from the disk. It depends mainly on molecular weight and solubility properties of the drug.

AGAR DILUTION

Many researchers use agar dilution method to decide the resistance of most pathogenic organisms to antibiotics. It is the commonly used dilution method to check the efficacy of new antibiotics.

Process

The agar is mixed with antibiotic to be evaluated and then it is added to dilution plates and diluted with different levels of water. Afterwards the disease causing organism to be tested is supplemented to each agar plate and an additional control plate that does not have any antibiotics. The dilution plates are incubated for sixteen to eighteen hours at a temperature of 37 degrees Celsius. Bacterial populations that divide quickly can be incubated for lesser time.

The plates are tested for bacterial growth after incubation. The extent of spread of bacteria that can be stopped by the lowest concentration of antibiotic is taken as the minimum inhibitory concentration of that bacteria⁶⁶.

Advantages

The most correct way to determine the antibiotic resistance of bacteria is Agar dilution method and it is the gold standard of susceptibility testing, many samples up to thirty pathogens can be tested at once. The results are easily monitored and can be reproduced.

Disadvantages

In agar testing every dilution plate infected by the pathogenic organism to be tested so agar dilution testing is cost effective and labour intensive. Agar dilution can be used to test only one antibiotic at a time but in broth micro dilution tests more than one antibiotic can be tested⁶⁷.

TREATMENT

Drug resistance in *Staphylococcus aureus* developed rapidly, so the antibiotics should be used judiciously to prevent resistance. For methicillin sensitive *Staphylococcus aureus* the first line drugs remain the treatment of choice, while for MRSA vancomycin and linezolid need to be given. Teicoplanin, daptomycin and quinupristin / dalfopristin could be alternate choices. Patients who colonise MRSA in the anterior nares should be treated with Mupirocin topical application twice a day for five days.

CONTROL MEASURES

To prevent hospital spread of *Staphylococcus aureus* hand washing is the most important step. Health care workers should be periodically screened for MRSA carriage and treated appropriately. Antibiotic usage should be restricted and preauthorization for high end antibiotics should be obtained prior to treatment. This will ensure misuse of antibiotics.

AIMS AND OBJECTIVES

- To detect phenotypic and genotypic resistance among *Staphylococcus aureus*
- Isolation and characterisation *Staphylococcus aureus* from clinical isolates.
- Disk diffusion and Agar dilution sensitivity methods for detection of Quinolone resistance.
- Detection of gyrase and topoisomerase genes from representative samples.

MATERIALS AND METHODS

STUDY POPULATION

Isolates obtained from various clinical samples received at the Diagnostic Microbiological laboratory, PSG Hospitals, Coimbatore.

STUDY LOCALE:

PSG Hospitals Coimbatore

STUDY PERIOD:

April 2016 - July 2017

SAMPLE SIZE ESTIMATION:

$$N = 4pq/d^2$$

Where n is required sample size p is expected prevalence q is 100-p and d is Degree of prevalence

SAMPLING METHOD:

Convenience sampling

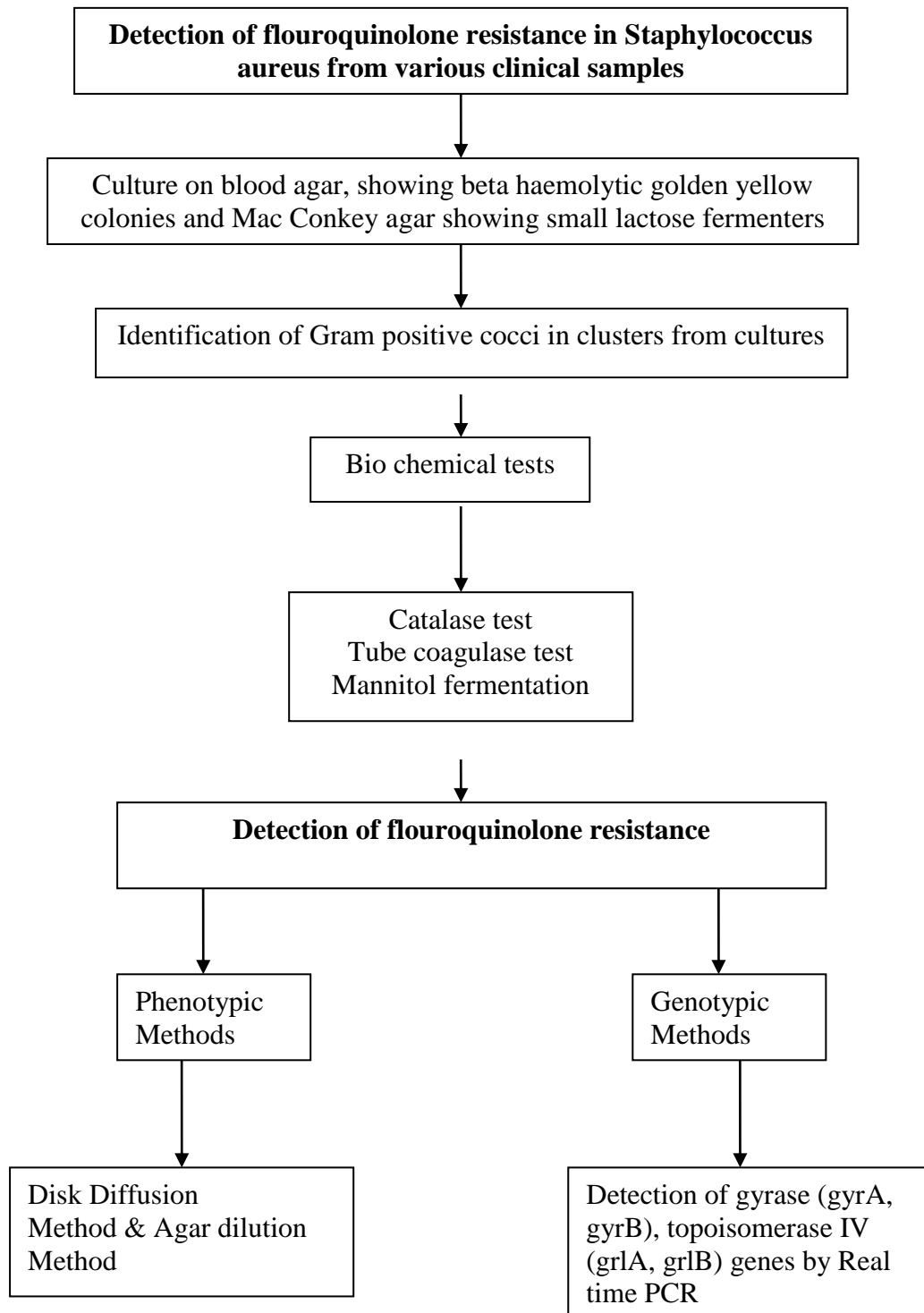
TYPE OF STUDY:

Cross sectional prospective study

ETHICAL CLEARANCE

This study was undertaken in the Diagnostic Microbiology Laboratory Department of Microbiology, PSG Hospitals. Institutional Human ethical Clearance was obtained, proof of which has been attached.

METHODOLOGY – FLOW CHART



SAMPLE COLLECTION:

All clinical samples which were sent to diagnostic microbiology laboratory were included in the study. Around 120 clinical isolates of *Staphylococcus aureus* from various clinical samples such as blood, wound swab, pus, sputum, broncho -alveolar lavage, tracheal aspirate, which grew beta haemolytic golden yellow colonies on 5 % sheep blood agar were processed.

SMEAR MICROSCOPY:

GRAM'S STAINING PROCEDURE:

- Slides were arranged and kept in serial order on the staining bridge one after another.
- Primary staining was done by crystal violet. The filtered stain was added on entire surface of the slide and kept for one minute.
- The slide was washed with water. In second step Lugol's iodine was added which act as mordant and kept for one minute.
- The slide was washed with gentle stream of water and was tilted to drain off excess water
- Decolourisation was done with Acetone with in ten seconds.
- The smear was counterstained by covering the entire surface of the slide with dilute carbol fuschin for thirty seconds

- Slide was rinsed with water to drain off the counter stain and under side of the smear was wiped.
- Slide was placed on the slide rack to air dry. After drying they were examined under microscope.

SMEAR EXAMINATION:

A drop of emulsion oil placed on the dried smear without touching the slide. The smears were examined with the 100 x oil immersion objective. Both positive and negative controls were put on the same slide. Positive controls from ATCC *Staphylococcus*, negative controls from ATCC *Escherichia coli* were satisfactory. Gram positive cocci in clusters were observed and identified as *Staphylococcus*.

CULTURE CHARACTERS:

All processing work was done in bio safety cabinet class II type A2 under strict aseptic precautions. Clinical samples were streaked on plates with 7% sheep blood agar and Mac Conkey agar. All culture plates were incubated at 35 to 37⁰ C up to 24 – 48 hours.

CULTURE EXAMINATION:

Cultures were examined after 48 hours of inoculation to detect growth. Blood agar plates were observed for the growth of beta haemolytic golden

yellow colonies. The Mac Conkey agar was examined for small lactose fermenting colonies without any contamination.

BIO CHEMICAL TESTS:

CATALASE TEST:

The presence of catalase enzyme was detected in the test isolate using hydrogen – peroxide.

Principle:

Catalase is an enzyme is produced by most of the aerobic and facultative anaerobic micro organisms to neutralize toxic forms of oxygen metabolites. It neutralizes bactericidal effects of hydrogen peroxide and protects the bacteria. This enzyme is absent in anaerobes.

The breakdown of hydrogen peroxide is mediated by catalase into oxygen and water. All the aerobic bacteria respire by using oxygen as a electron acceptor.

Procedure of Catalase Test: (Slide test)

1. A loop or sterile wooden stick was used to transfer a small amount of bacterial colony to the surface of dry, clean glass slide.
2. A drop of 3% H_2O_2 was placed on the slide and mixed with the bacterial colony.

3. A positive result was interpreted by rapid evaluation of oxygen within 5 – 10 Seconds as evidenced by bubbling.
4. The slide was disposed in the biohazard disposal container.

Procedure of tube Catalase Test:

1. A test tube was added with 4 to 5 drops of 3 % H₂O₂.
2. A small amount of organism was transferred to the test tube with wooden applicator.
3. The test tube was placed against dark background and observed for immediate bubble formation at end to wooden applicator.

TUBE COAGULASE TEST:

This is used to differentiate between *Staphylococcus aureus* and coagulase negative *Staphylococcus* CONS.

Principle of the Test

The staphylocoagulase produced by the *Staphylococcus aureus* reacts with coagulase reacting factor which is a thrombin like molecule. They both combine to indirectly convert fibrinogen to fibrin and gives positive result.

Procedure:

Human plasma was diluted in saline as 1 in 6 dilutions and one ml volume of this diluted plasma was placed in small tubes. Several isolated colonies of

Staphylococcus aureus were emulsified in the plasma to get a milky suspension. All tubes were incubated at 37⁰ C for 4 hours. All tubes were examined for clot formation by tilting the tube at 90⁰ at end of 4 hours incubation. The negative tubes were left at room temperature over night and re-examined. This step was essentially done because some strains including many MRSA produce a delayed clot which was rapidly lysed by staphylokinase produce by organism. The positive result was interpreted by clot formation. Appropriate positive and negative controls were put.

ANTIBIOTIC SENSITIVITY PROCEDURE:

Kirby Bauer disc diffusion:

The peptone water was inoculated with the test organism and incubated for four hours at 37⁰C. The turbidity was matched with 0.5 Macfarlands. A sterile swab was soaked in the inoculum and squeezed against the wall of the test tube and was spread over the Muller – Hinton agar plate. Antibiotic discs like Vancomycin (30 µg), linezolid (30 µg), Amoxy-clav (30 µg), Clinadamycin (2 µg), Erythromycin (15 µg), Penicillin (6 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Ofloxacin (5 µg) were placed on the MHA plate and tested for sensitivity pattern. A cefoxitin 10 µg disc was placed over the MHA plate and incubated at 37 ⁰C for 24 hours. Next day zone size was measured. A zone size of > 22mm was interpreted as Methicillin sensitive *Staphylococcus aureus* (MSSA) and less than 22 mm was considered as Methicillin resistant *Staphylococcus aureus* (MRSA)

The zone diameter of fluoroquinolones like Ciprofloxacin, Ofloxacin.

Levofloxacin with appropriate disc strength was interpreted as follows

FLUOROQUINOLONES *Staphylococcus* species.

| Antimicrobial agent | Disk content | SENSITIVE | INTERMEDIATE | RESISTANT |
|---------------------|--------------|-----------|--------------|-----------|
| Ciprofloxacin | 5µg | ≥21 | 16-20 | ≤15 |
| Levofloxacin | 5 µg | ≥19 | 16-18 | ≤15 |
| Ofloxacin | 5 µg | ≥18 | 15-17 | ≤14 |

CLSI guidelines for Antimicrobial susceptibility testing – 2016

Testing of MIC by Agar Dilution Method.

MIC was done by agar dilution as per the (CLSI-2013) guide lines. The materials used for the agar dilution were Mueller Hinton agar, Pure pharmaceutical products of antimicrobial powder, petriplates, pipettes, antimicrobial solvents, disposable tips, sterilized test tubes etc. The antimicrobial agents used for testing were Ciprofloxacin, Levofloxacin, and ofloxacin for the species *Staphylococcus aureus*.

Preparation of drug dilutions

The drug dilutions are prepared according to the CLSI guide lines (M26-A) by agar dilutions methods. The table describes drug dilution protocol as per CLSI Guidelines

Preparation of stock solution of antibiotic solution

Stock solutions are generally prepared by using this formula:

$$\frac{1000}{P} \times V \times C = w$$

Where

P= potency given by the manufacturer in relation to the drug

V= volume (ml) required

C= final concentration of the solution

W= weight of the antibiotic to be dissolved in the required volume.

SCHEMATIC DRUG DILUTION

| Drug and step no. | Concentration (µg/ml) | Source | Volume (ML) | Diluents | Intermediate concentration | Final concentration 1ml Mixed with 20 ml |
|-------------------|-----------------------|--------|-------------|----------|----------------------------|---|
| 1 | 5120 | Step 1 | 1ML | 3 ML | 1280 | 64 |
| 2 | 1280 | Step 1 | 1ML | 1ML | 640 | 32 |
| 3 | 640 | Step 2 | 1ML | 1ML | 320 | 16 |
| 4 | 320 | Step 3 | 1ML | 1ML | 160 | 8 |
| 5 | 160 | Step 4 | 1ML | 1ML | 80 | 4 |
| 6 | 80 | Step 6 | 1ML | 1ML | 40 | 2 |
| 7 | 40 | Step 7 | 1ML | 1ML | 20 | 1 |
| 8 | 20 | Step 8 | 1ML | 1ML | 10 | 0.5 |

PREPARATION OF INOCULUM:

The inoculums were prepared from fresh subculture of *Staphylococcus aureus* isolates by using a sterile inoculation loop by picking 4 to 5 similar colony in to the sterile saline.

The suspensions were matched to McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/ml). The working dilutions were made further by diluting 25 times in to CAMHB (Cation-adjusted Mueller-Hinton broth) to obtain a final organism concentration of 3×10^5 to 5×10^5 CFU/ml in each.

Procedure:

- The drugs diluted were prepared from higher to lower as mentioned in the table in the test tubes. This diluted drug solutions were mixed in to 20 ml of media. The final concentrations were achieved 64µg/ml after mixing in to 20ml of media. Similarly other concentrations also made to achieve 32µg, 16µg, 8µg, 4µg, 2µg, 1µg, 0.5µg as follows.
- Drug free control plates were prepared without adding drug in to the medium.
- Before inoculation of culture in to the medium the plates was divided Column and Marked for identity on backside of plate.
- 1µl of inoculum that were transferred from prepared inoculum of 10^4 cfu /ml by dilution in to medium of appropriate Column.

- Different isolates were inoculated in to different labeled Column.
- The plates were incubated at 37 °C for 18- 20 hours.

Interpretation of MIC:

- The results were interpreted by checking Growth on drug free medium, control plate showing satisfactory growth.
- The MIC was calculated by the inhibition of visible growth in least concentration of each isolate in particular concentrations were considered MIC of Each isolate.

| Antimicrobial agent | MIC(μg/ml) SENSITIVE | MIC(μGg/ml) INTERMEDIATE | RESISTANT |
|----------------------------|--|--|------------------|
| Ciprofloxacin | ≤ 1 | 2 | ≥ 4 |
| Levofloxacin | ≤ 1 | 2 | ≥ 4 |
| Ofloxacin | ≤ 1 | 2 | ≥ 4 |

CLSI guidelines for Antimicrobial susceptibility testing – 2016

Genotypic Method for detection of Fluroquinolone resistance

The DNA purification procedure using the miniprep spin columns comprises of three steps

1. Adsorption of DNA to the membrane.
2. Removal of residual contaminants.
3. Elution of pure genomic DNA.

Principle

Bacterial cells are grown in a medium till they reach log phase and are harvested by centrifugation. The bacterial (Gram positive) cell wall is degraded

by lysozyme and Proteinase K. For Gram negative bacteria, the lysozyme treatment is not required. Following lysis, the DNA is bound to the silica-gel membrane of the HiElute Miniprep Spin Column (Capped) to yield approximately upto 20µg of pure DNA. Two rapid wash steps remove trace amount of salt and protein contaminants resulting in the elution of pure DNA in the Elution Buffer provided with the kit.

Reagents provided (HiPurA Bacterial Genomic DNA Purification Kit)

Gram Positive Lysis Solution (GPLA)

Lysis Solution (AL)

Lysis Solution (C1)

Prewash Solution (PWB)

Wash Solution Concentrate (WS)

Elution Buffer (ET) [10 m M Tris - Cl, pH 8.5]

Proteinase K

RNase A Solution (20 mg/ml)

Lysozyme

HiElute Miniprep Spin Column (Capped)

Collection Tube(Uncapped) , Polypropylene (2.0 ml)

Collection Tube, Polypropylene (2.0 ml)

Procedure:

1. An overnight bacterial broth culture was pelleted in to a 2 ml capped collection tube of about 1.5 ml and was centrifuged for 2minutes 13,000 rpm at room temperature (15 – 25⁰C).

2. 200µl of Lysozyme Solution was added for each isolate and
Incubate at 37°C for 30 minutes.
3. The Pellet was resuspended thoroughly in 180µl of Lysis Solution.
4. 20µl of the Proteinase K solution was added to the sample mixed and
was incubated for 30 minutes at 55°C.
5. 200µl of Lysis Solution (C1) was added vortexed thoroughly (about
15 seconds) and incubated at 55°C for 10 minutes.
6. 500µl of prewash Solution was added to the column and centrifuged
at (≈10,000 rpm) for 1 minute at room temperature.
7. 500µl of diluted Wash Solution (WS) was added to the column and
centrifuged for 3 minutes at 13,000 rpm at room temperature (15-
25°C). Discarding the flow through was done and has been spin
again at same speed for the additional 1 minute to dry the column.
8. Transferring of the HiElute Miniprep Spin Column (Capped) to fresh
uncapped collection tube was done. Pipette 200µl of the Elution
Buffer (ET) directly into the column without spilling to the sides and
incubated for 1 minute at room temperature and then centrifuged at
(≈10,000 rpm) for 1 minute at room temperature (15-25°C) to elute
the DNA.
9. Transferring of the eluate to a fresh capped 2ml collection tube was
done for longer storage at – 20°C.

DNA Amplification

The extracted DNA from the isolates were amplified by using Real time PCR = AB Applied bio systems

Sterile 1.5 ml tubes were taken for the preparation of PCR

Master mix = 12.5 Microlitres, PCR water 8.5 Microlitres

DNA lysate 2 Microlitre

| Target | Primer | Sequence 5' to 3' | Expected Amplicon size(bp) |
|-------------|----------------|---------------------------------------|--------------------------------------|
| <i>grlA</i> | Forward primer | 5'-TGC CAG ATG TTC GTG ATG-3' | 5'nucleotide at position 2467) |
| | Reverse | 5'CCT TGA ATA ATA CCA CCA GTT G-3' | (5' nucleotide at position 3040) |
| <i>grlB</i> | Forward primer | 5'-TGT TGT GTC TGT TCG TAT TCC-3' | (5' nucleotide at position 1353) |
| | Reverse primer | 5'-GCA CCA TCA GTA TCA GCA TC-3' | (5' nucleotide at position 1910) |
| <i>gyrA</i> | Forward primer | 5'-GAG TGT TAT CGT TGC TCG TG-3' | (5' nucleotide at position 2333) |
| | Reverse primer | 5'-GAC GGC TCT CTT TCA TTA CC-3' | (5' nucleotide at position 2725) |
| <i>gyrB</i> | Forward primer | 5'-CCA CAA GTC GCA CGT ACA G-3' | (5' nucleotide at position 1407) |
| | Reverse primer | 5'-ATC CAC ATC GGC ATC AGT C-3' | (5' nucleotide at position 1817). |

The temperature setup was done for the following steps

Holding time 95° C for 5 minutes

Denaturation 95°C for 30 seconds

Annealing 46°C for 45 seconds

Elongation 72° C for 59 minutes

Final elongation 72°C for 7 minutes

At the end of 30 cycles the amplified products were obtained.

GEL ELECTROPHORESIS

1. Sterile beaker was taken, 1x buffer was prepared, and 80 ml of 1x buffer was added with 0.76 grams of agarose powder and then kept in the micro wave oven for melting for 3 minutes.
2. After 3 minutes the agarose was cooled to 40°C, to this 2 micro litre of Ethidium bromide was added.
3. In 80 ml trough the agarose gel was poured with 15 numbered comb to make wells. After the agar gets solidified the comb was taken out.
4. In the first well the 100 base pair ladder was added to the rest of the wells the amplified DNAs were added.
5. The gel was run at 95 volts for 45 minutes and the appearance of bands were noted under UV light.
6. The bands at 550 base pairs indicates *grlA* gene.
7. The bands at 463 base pairs indicates *grlB* gene.
8. The bands at 373 base pairs indicates *gyrA* gene.
9. The bands at 392 base pairs indicates *gyrB* gene.

RESULTS

A total of 24,024 samples of pus, blood, wound swab, tracheal aspirate and urine were processed in microbiology laboratory between April 2016 to July 2017. The incidence of *Staphylococcus aureus* from the samples were 3.5%.

Figure: 1 shows the distribution of *Staphylococcus aureus* that were resistance to fluoroquinolones was about 120 among the total 859 isolates received from various samples. It was found to be 14.3%

Figure: 2 depicts the distribution of *Staphylococcus aureus* from various clinical samples received which was most commonly isolated from pus sample 72%, followed by 14% from blood 8% from tracheal aspirate and 6% from urine sample

Illustration 1: Gram's stain picture shows the gram positive cocci in clusters.

Illustration 2. Growth of *Staphylococcus aureus* as beta haemolytic golden yellow colonies on blood agar.

Growth on MacConkey agar showing small lactose fermenting colonies in

Illustration 3.

Biochemical tests to differentiate *Staphylococcus aureus*. e.g. Tube coagulase, Mannitol with both positive and negative controls are shown in **Illustrations 4 & 5.**

Antibiotic sensitivity was performed by Disc diffusion and Agar dilution methods **Illustration 6 & 7.**

Table 1 depicts the pattern of susceptibility for the isolates of *Staphylococcus aureus* for various antibiotics.

Figure : 3 shows the pattern of fluroquinolones resistance among the isolates of *Staphylococcus aureus*.

Table 2 depicts the MIC for the fluroquinolones resistant *Staphylococcus aureus*.

The molecular characterisation for the *gyrA* *gyrB* *grlA* *grlB* was done by Real time PCR and amplification by gel electrophoresis is shown in **Illustrations 8, 9 & 10**.

The expression of *grlB* and *gyrB* was found in 24% of isolates which is depicted in **Illustration 11**.

Figure : 1 Distribution of fluroquinolones resistance among total number of *Staphylococcus aureus*

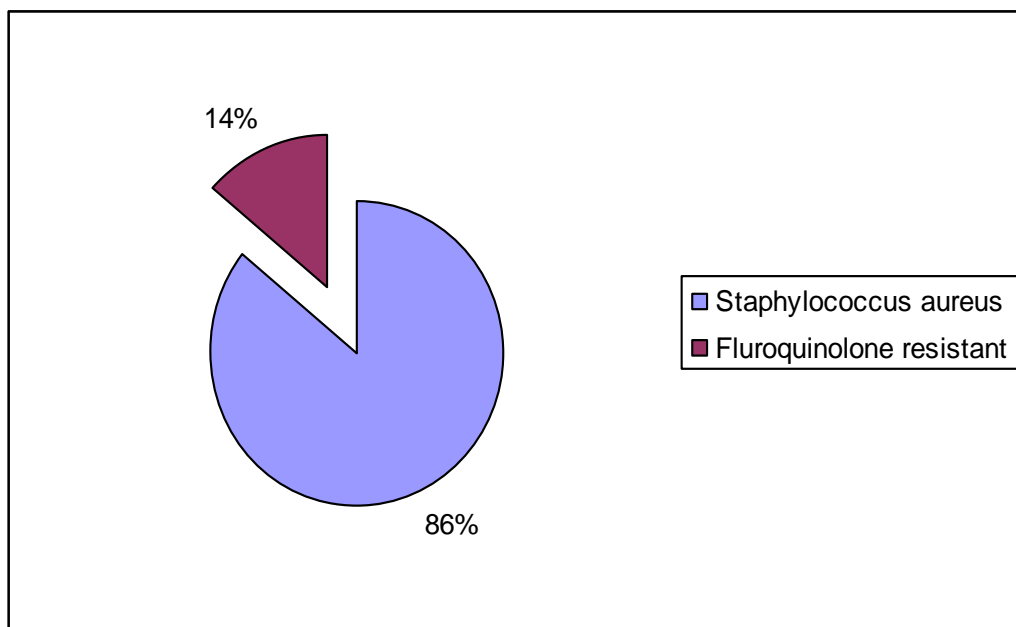


Figure : 2 Distribution of *Staphylococcus aureus* from various clinical samples.

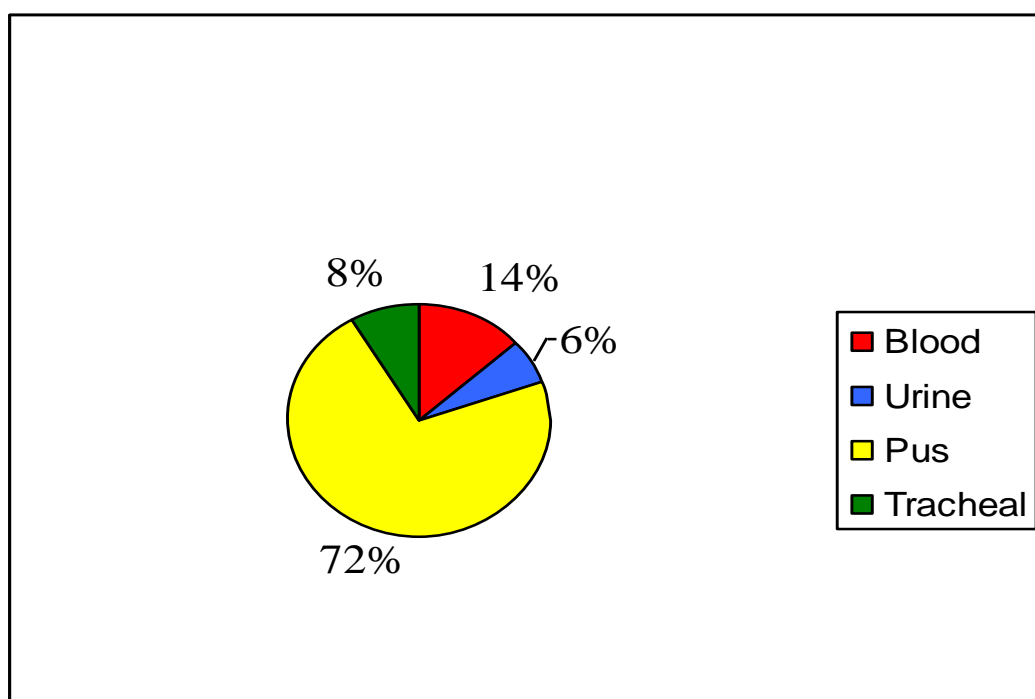


Illustration 1: Gram's stain shows the gram positive cocci in clusters.

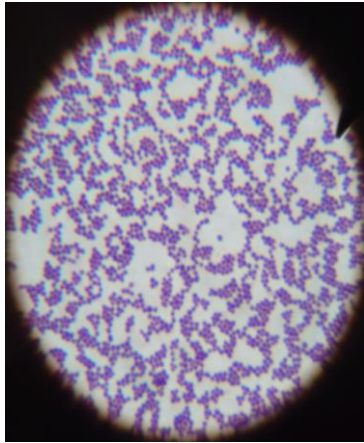


Illustration 2: Beta haemolytic golden yellow colonies on blood agar.



Illustration 3: Small lactose fermentors on MacConkey agar



Illustration 4: Tube coagulase and mannitol fermentation



Illustration 6: Antibiotic sensitivity by Disc diffusion

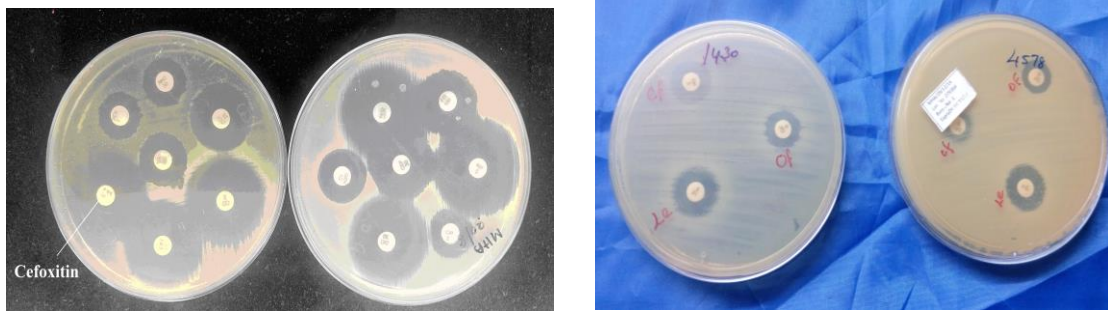


Illustration 7: Agar dilution method

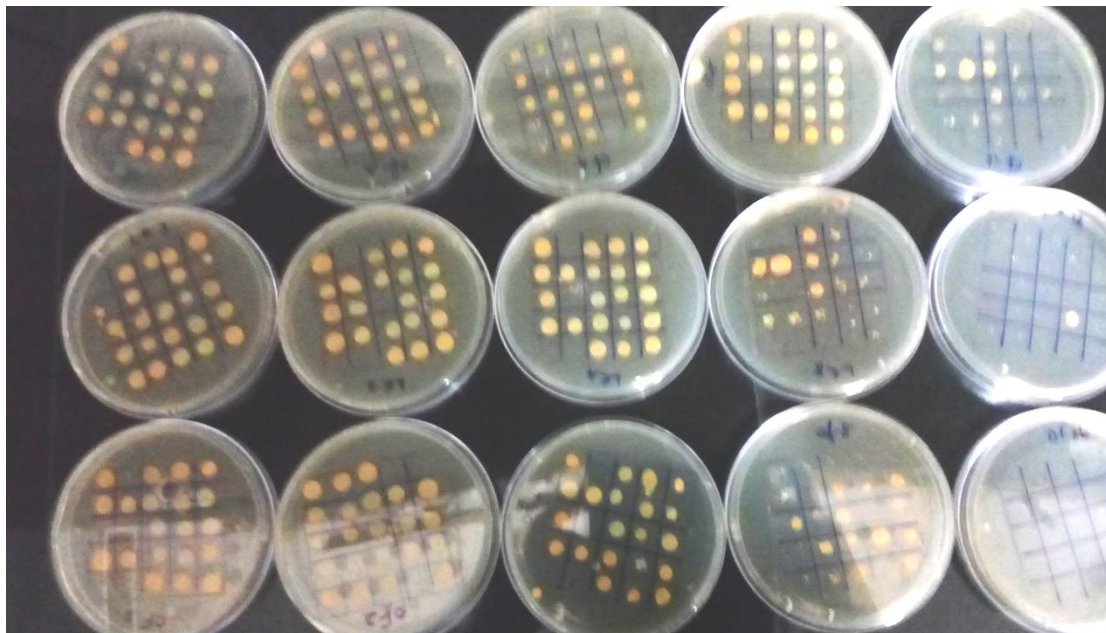


Table 1. Pattern of susceptibility of the isolates of *Staphylococcus aureus* for various antibiotics.

n= 120

| Antibiotic (µg) | Sensitive (%) | Resistance (%) |
|-------------------------|-----------------------|------------------------|
| Vancomycin (30 µg) | 100 | 0 |
| Linezolid (30 µg) | 100 | 0 |
| Amoxy-clav (30 µg) | 74 | 26 |
| Clinadamycin (2 µg) | 88 | 12 |
| Erythromycin (15 µg) | 69 | 31 |
| Penicillin (6 µg) | 13 | 87 |
| Ciprofloxacin (5 µg) | 0 | 100 |
| Levofloxacin (5 µg) | 0 | 100 |
| Ofloxacin (5 µg) | 0 | 100 |
| Cefoxitin(10 µg) | 37 | 73 |

Table 2 : Minimum Inhibitory Concentration (MIC) for the Fluroquinolone resistant *Staphylococcus aureus*.

| Antibiotic | MIC 8 - 16µg | MIC 16 - 32 µg | MIC 32 - 64 µg |
|---------------|--------------|----------------|----------------|
| Ciprofloxacin | 52 | 38 | 30 |
| Levofloxacin | 16 | 45 | 39 |
| Ofloxacin | 18 | 43 | 49 |

Figure 3 : Pattern of Fluroquinolone resistance in MSSA & MRSA.

n= 120

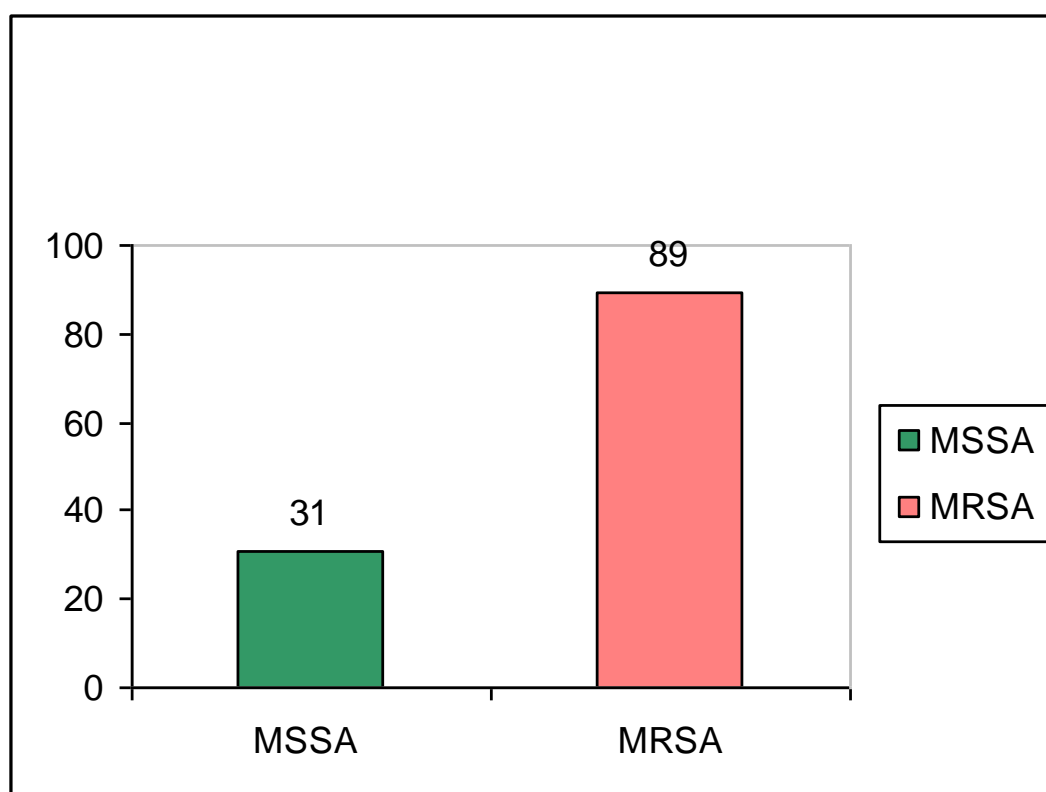


Illustration 8: DNA Amplification (Thermocycler)



Illustration 9: Gel electrophoresis



Figure : 4 Shows Distribution of Flouroquinolone resistant genes among test isolates

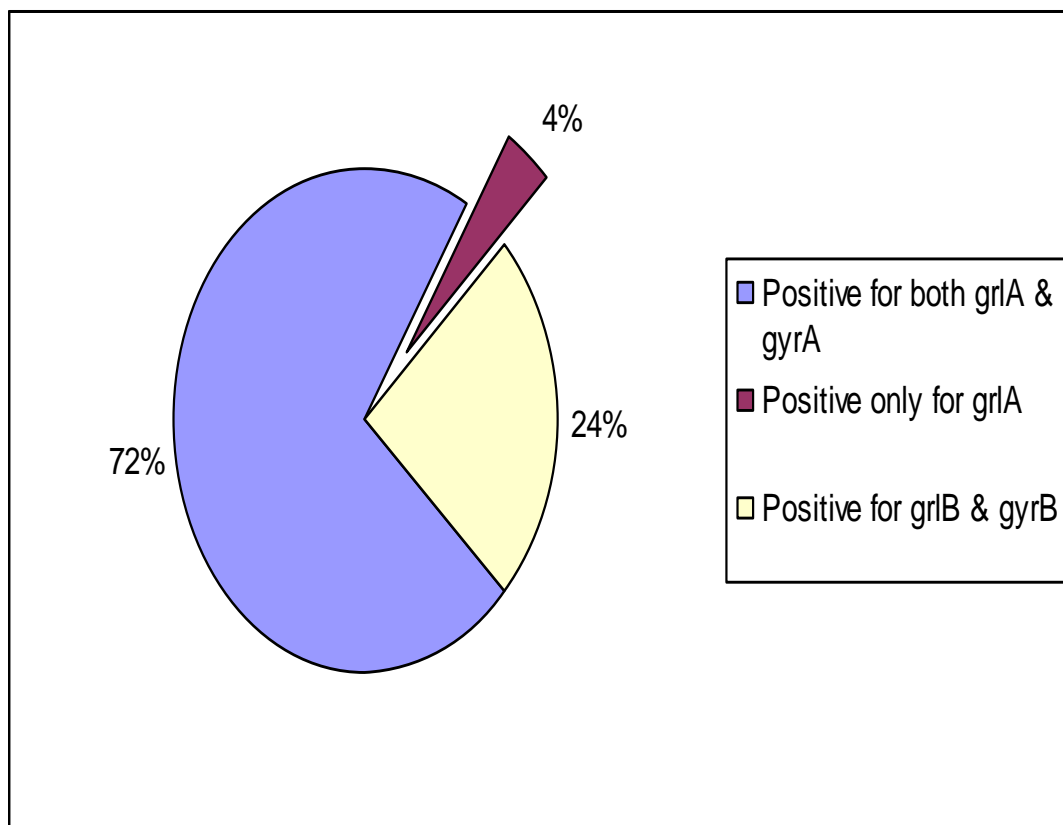


Illustration 10: The molecular characterisation for the *gyrA* *gyrB* *grlA* *grlB* done by Real time PCR.

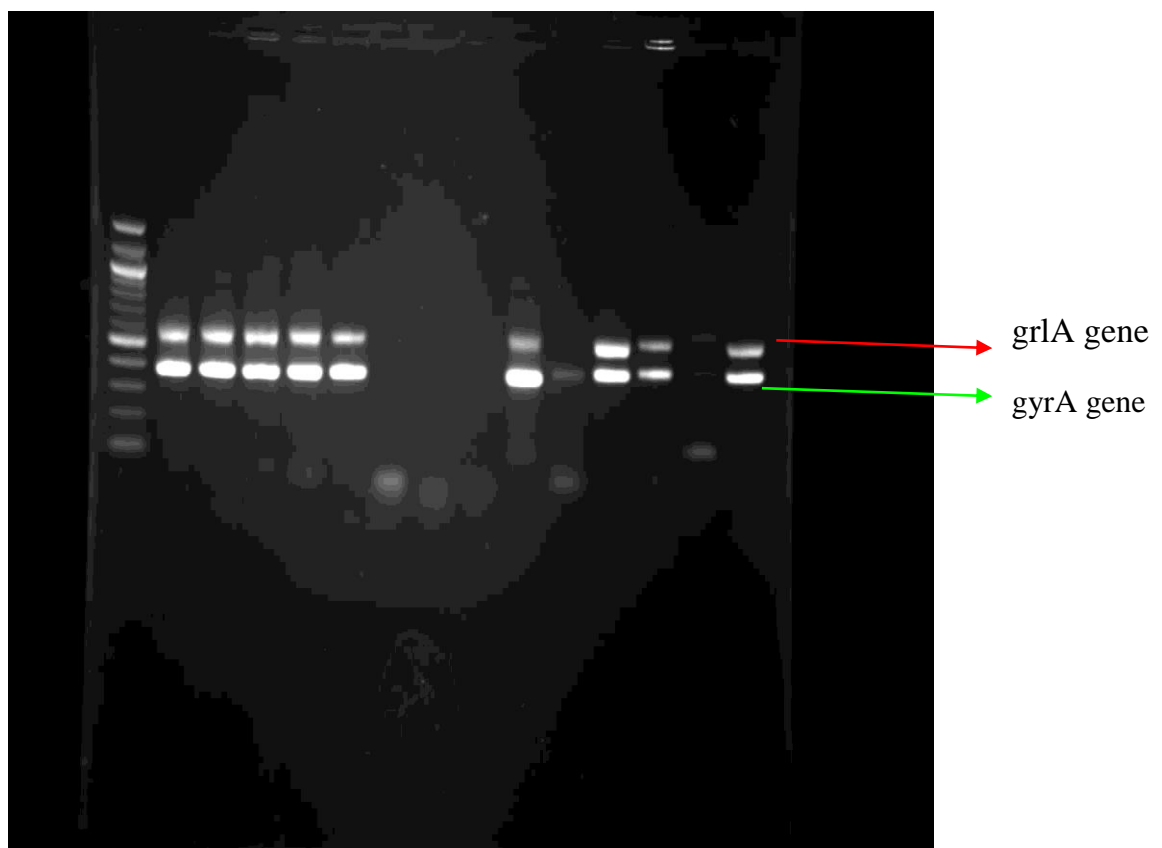
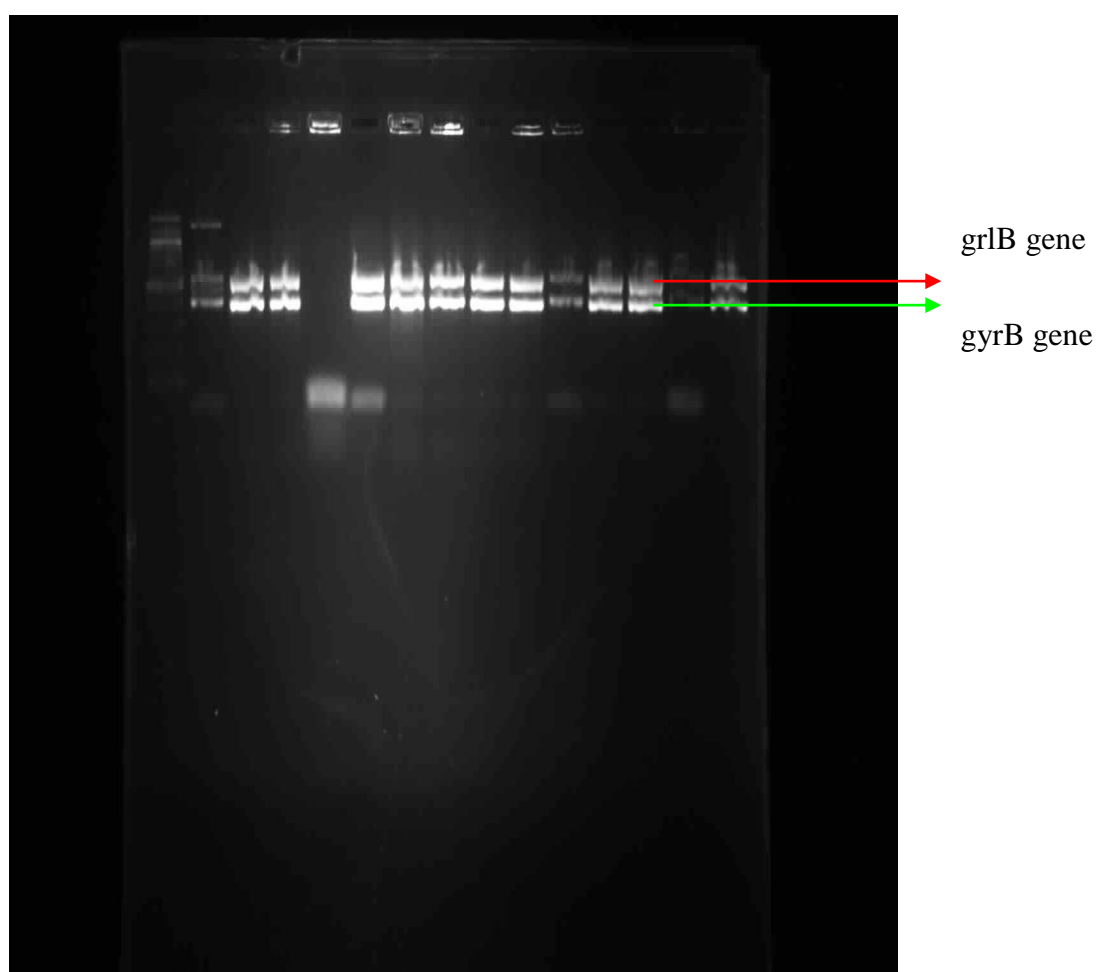


Illustration 11: Expression of *grlB* and *gyrB* genes among isolates of *Staphylococcus aureus*



DISCUSSION

Staphylococcus aureus constitutes an important pathogen and is found to be colonised in approximately 30% of the population who are asymptomatic. Drug resistance has evolved due to increasing inadvertent use of high end antibiotics and the incidence of multidrug resistant bacteria is emerging rapidly.

The incidence of MRSA in our study was found to be 74%. This correlates well with other studies in and around world. MRSA isolates reported across various parts of the world range between 28 % to 87%.⁶⁸

Literature surveys reveal that the high percentages of isolates are of MRSA are found to be resistant to fluroquinolones as compared to MSSA. In our study 74% of MRSA were resistant to fluroquinolones among which most predominant was 43.3% to ciprofloxacin (43.3%) followed by ofloxacin (37.5%) and levofloxacin (34.2%).

MRSA colonisation and infection in patients relates to the effect of fluroquinolones and cotrimoxazole. Among the isolates of MRSA tested , in one study from patients 74% resistant to ciprofloxacin and 68% were resistant to cotrimoxazole were noted. In our study ,among clinical isolates of MSSA, 26% were resistant to ciprofloxacin and 32% were resistant to cotrimoxazole.

High prevalence of MRSA (64%) was observed from pyogenic infections followed by blood stream infections. In a study in New Delhi high prevalence of MRSA 35% in ward and 43% in ICU was observed from blood culture specimens⁶⁹.

Anti microbial susceptibility testing of isolates of *Staphylococcus aureus* showed maximum sensitivity to vancomycin (100%) , linezolid (100 %) followed by clindamycin(88%) amoxyclav(74%)..Highest resistance was noted for ciprofloxacin followed by ofloxacin.

First generation cephalosporins form the main stay of treatment for MSSA infections in patients intolerant to anti staphylococcal penicillins. If the patient has been empirically started on vancomycin and the culture report reveals MSSA de-escalation to beta lactams is the preferred mode of treatment. Glycopeptides- vancomycin and teicoplanin should be used only as a last resort and reserve drug.⁷⁰

The minimum inhibitory concentration susceptibility profile to fluoroquinolones was tested by agar dilution method. Among the 120 isolates of *Staphylococcus aureus* tested MIC value 32 to 64µg for ciprofloxacin, 16 to 32µg for Ofloxacin and 8 to 16µg for Levofloxacin. The range of MIC value for fluoroquinolones was between 32 to 64µg for MRSA as against 8 to 16µg for MSSA.

Studies on ocular infections have reported ciprofloxacin resistance ranging from 3 to 11% as compared to Levofloxacin 25.5%. Increasing resistance among *Staphylococcus aureus* isolates (MSSA) for ciprofloxacin and levofloxacin poses a threat for management of infections as treatment choices are narrowed.⁷¹

In another study fluoroquinolones resistant rate of 41.8% was found among *Staphylococcus aureus* isolates which was comparatively higher than similar reports which showed resistance range between 13% and 20.7%^{72, 73}

The mechanism of genotypic resistance to fluoroquinolones in *Staphylococcus aureus* has been extensively studied. In majority of isolates single mutations in DNA topoisomerase IV and DNA gyrase have been reported. DNA topoisomerase IV is the primary target of fluoroquinolones in *Staphylococcus aureus* with the first step to these agents being mutations in *grlA*.⁷⁴

In our study a representative population of fifty isolates of resistant *Staphylococcus aureus* by both disk diffusion and agar dilution were subjected to real time PCR assay for detection of topoisomerase iv and gyrase. The results of our study showed majority of the isolates (72%) of *Staphylococcus aureus* were detected to express *grlA* and *gyrA*. This correlates well with other studies which report similar results.⁷⁵

There have been reports showing one or two point mutations in the QRDR regions of both *grlA* and *gyrA* genes to be the main mechanism of ciprofloxacin resistance among isolates of *Staphylococcus aureus*.⁷⁵ as shown by pulsed-field gel electrophoresis studies.

We noted high level ciprofloxacin resistance for 43.3% of the isolates tested (MIC 32-64 µg /ml) This level of high resistance is usually associated with *grlA* and *gyrA* mutations. This finding also correlates well with other studies.⁷⁴

Though other molecular methods for detection of fluroquinolones exist eg: Restricted fragment length polymorphism (RFLP), High performance liquid chromatography (HPLC) and gene sequencing, they are cumbersome and labour intensive. The advantages of Real time PCR include high sensitivity and the turnaround time is far less compared to other methods. In laboratories which have facilities for molecular testing of Real time PCR could be used as a routine diagnostic tool.

CONCLUSION

Injudicious use of antibiotics has led to widespread emergence of resistant organisms. Newer compounds have been introduced in the market but inadvertent use of these drugs by clinicians have left us with resistant bugs which are very difficult to eradicate. The strict implementation of antibiotic policy tailor made to the individual hospital needs and prompt regular audits of compliance to the same are the only means of saving the populations from newer pathogens and multi drug resistant bugs.

SUMMARY

- Among the isolates of *Staphylococcus aureus* the incidence of MRSA was found to be 74%.
- The resistance to fluoroquinolones among the total isolates of *Staphylococcus aureus* was 14%.
- High level of ciprofloxacin resistance was noted among majority of the isolates tested (MIC: 32 to 64 µg ml).
- MIC to levofloxacin and ofloxacin ranged between (8 to 16 µg and 16 to 32 µg) respectively.
- Real time PCR for detection of fluoroquinolone resistance showed *grl A* and *gyr A* to be the most predominant genes expressed. (72%).
- A small proportion (24%) expressed *grl B* and *gyr B*

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ANNEXURES

Annexure I –Ethics Clearance form

ANNEXURE - II

PREPARATION OF REAGENTS

BLOOD AGAR:

Sterile defibrinated sheep blood 7ml

Nutrient agar (melted) 100ml

About 7ml of melted nutrient agar ,was poured as a base ,into sterile Petri dishes and allowed for setting. This forms a thin base for pouring in the blood agar.Sterile defibrinated sheep blood (5-7%) was added to nutrient agar, the latter should be cooled to about 45-50⁰c before blood is added.It was mixed well and about 15ml of blood agar was poured over the base in each Petri dish.

MAcCONKEY AGAR:

Peptone - 2.0gm

Sodium chloride – 0.5gm

Bile salt - 0.5gm

Lactose - 1.0gm

Agar - 1.5gm

Distilled water - 100ml

The ingredients except lactose were dissolved in distilled water by heating. pH was adjusted to 7.6 .1ml of 1% neutral red solution was added to every 100ml of medium with lactose. Sterilized by autoclaving at 121⁰c for 15 minutes.

MUELLER-HINTON AGAR

| | |
|-------------------|----------|
| Beef extract | - 2.0gm |
| Acidicase peptone | - 7.5gm |
| Starch | - 1.5gm |
| Agar | - 17.0gm |
| Distilled water | - 1000ml |

The ingredients were dissolved in one litre of distilled water and mixed thoroughly. Heat with frequent agitation and boil for one minute. pH was adjusted to 7.4 +/-0.2. Sterilized by autoclaving

MANNITOL FERMENTATION MEDIUM:

SUGAR SOLUTION:

| | |
|---|---------|
| Sugar (Mannitol) | - 0.5gm |
| Nutrient broth base | - 100ml |
| Bromothymol blue indicator(0.2% alcoholic)- | 1.2ml |

Sugar solution was prepared as described above. It was dispensed in 2-4ml quantities into test tubes (12x100mm) Autoclaving was done at 115⁰c for 10 minutes.

Annexure III – Plagiarism Check

URKUND

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Submitted by S.Geethanjali (geethavelurs@gmail.com)

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frequently found in the nose, respiratory tract, and on the skin.

Although Staphylococcus aureus is not always pathogenic, it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning. Infections are produced by virulent strains that induce potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies.

Staphylococcus aureus can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases

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1 Warnings

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